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Insights into The Ecology, Genetics and Distribution of Lucanus Elaphus Fabricius (Coleoptera: Lucanidae), North America's Giant Stag Beetle

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Insights into the ecology, genetics and distribution of *Lucanus elaphus* Fabricius (Coleoptera: Lucanidae), North America's giant stag beetle

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Abstract. 1. Little is known about the biology or conservation status of *Lucanus elaphus* Fabricius in North America despite well-documented declines of a related species, *Lucanus cervus* (L.), in Europe. This study provides information critical to developing conservation plans for *L. elaphus* including the species' larval substrate requirements, genetic data and range-wide estimates of habitat suitability.

2. In Mississippi floodplain forests, larval *L. elaphus* were recovered from a wide range of log sizes and rot types and were either found tunnelling within the wood or feeding beneath logs at the soil–wood interface. The species appears to require 1–2 years to complete development, exhibits a 1:1 sex ratio and is parasitised by *Zelia vertebrata* (Say) (Diptera: Tachinidae).

3. Flight intercept traps placed at three heights at both the edge and interior of hardwood-dominated forests in Georgia yielded six adult male *L. elaphus*, all of which were captured in traps placed at 15 m on the forest edge.

4. Because *L. elaphus* larvae are morphologically indistinguishable from related species, DNA sequence data from the mitochondrial cytochrome oxidase I gene were generated to facilitate molecular identification. Genetic data revealed modest intra-specific variation, with up to 1.3% sequence divergence among haplotypes sampled from the same forest.

5. Based on assembled occurrence records, ecological niche models suggest that environmental conditions are suitable for *L. elaphus* across much of the southeastern United States, provided that adequate lowland forest cover and dead wood substrates are available.

Key words. Conservation, decomposing wood, ecological niche model, forest biodiversity, saproxylic.

Introduction

Relatively little is known about the diversity, ecology or conservation status of saproxylic insects in North America (i.e. those dependent on dead or dying wood). This is true even for some of the largest and most striking species, such as the giant stag beetle, *Lucanus elaphus* Fabricius (Fig. 1a, b). Published information on *L. elaphus* has focused mostly on the species' distribution, notable collection records or aberrant specimens (Wickham, 1903; Staines, 1994, 2000, 2001; Ratcliffe & Christen, 2002). Most specimens (primarily males) have been collected at

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electric lights (Staines, 2001), although adults are also sometimes observed feeding on sap flows and are thought to breed in decomposing logs or stumps (Blatchley, 1910). There is particular interest in research on *L. elaphus* given that a closely related species native to Europe, *Lucanus cervus* (L.), has become threatened throughout much of its range (Campanaro *et al*., 2016). There are likely to be parallels in the threats facing *L. cervus* in Europe and those potentially facing *L. elaphus* in North America. These may include logging and/or harvesting of downed woody debris, expansion and intensification of agriculture and urbanisation (Nieto & Alexander, 2010). With the goal of providing baseline data to inform strategies for the conservation of *L. elaphus*, here we present information on the ecology, genetics and geographic distribution of the species.

Insect conservation requires knowledge of larval habitat associations (Foit *et al*., 2016). However, such information is extremely limited or non-existent for many stag beetles, including species of *Lucanus* (Huang, 2014). Previous studies suggest that lucanids may not be very discriminating with respect to their substrate requirements. For instance, *L. cervus* larvae have been reported from over 60 different species of host tree in Britain as well as from unexpected substrates like railway timbers, fence posts and compost heaps (Harvey *et al*., 2011a). Moreover, substrate associations of *L. cervus* vary across Europe, possibly reflecting differences in resource availability among regions (Harvey *et al*., 2011a). Other stag beetle species are similarly accepting of a broad range of breeding substrates. In Tasmania, for example, *Lamprima aurata* (Latreille) has been reported from a diverse assortment of tree species as well as from buried wood near house foundations, fence posts, telegraph poles and piles of sawdust (Fearn, 1996). Specific substrate associations have been reported for some stag beetles, however. In Japan, Ikeda (1987) reported that three species of *Platycerus* exhibit preferences for particular tree species, log diameters, moisture content and carbon/nitrogen ratio. Also in Japan, Araya (1993a,b) found that some lucanid species associate with particular rot types or log diameters, however, most species showed no clear patterns of substrate use. Niche partitioning has been reported for the lucanid fauna of northern Australia, with different species preferring white rot, brown rot, drier substrates, sapwood, lower elevation, etc. (Wood *et al*., 1996). Indeed, fungal species appear to be more important than host tree for many stag beetles, for example, *Phalacrognathus muelleri* (Macleay) utilises a wide range of wood types provided that suitable white rot fungi are present (Wood *et al*., 1996). There are 24 species

Fig. 1. *Lucanus elaphus* adult males (a) and female (b). Larvae were collected from a wide range log sizes, rot types and within-log locations. Some larvae were found feeding at the soil–wood interface (c, arrows), whereas others were found feeding within relatively sound wood (d) or within wood highly degraded by white (e) or brown (f, arrow) rot. [Colour figure can be viewed at wileyonlinelibrary.com]

of Lucanidae in North America, but very little is known about their larval resource requirements. A major focus of this study was therefore to learn more about habitats utilised by larval *L. elaphus*. We also report data on captures of adult *L. elaphus* in flight intercept traps to better understand the species' flight behaviour and season of activity.

For many insect species, using morphological features to identify specimens at immature stages can be challenging, if not impossible. Previous researchers have determined that larval *L. elaphus* cannot be distinguished from other North American species of *Lucanus* (Ritcher, 1966), making it necessary to raise all collected larvae to adulthood. To facilitate identification of *L. elaphus* specimens at any life stage, we sequenced a 1364-bp fragment of the mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I (*COI*) gene, this fragment includes 579 bp of the widely used 658-bp 'DNA barcoding' region (Hebert *et al*., 2003), facilitating comparison with many other species in public DNA sequence databases. If *L. elaphus COI* sequences are sufficiently different from those of other *Lucanus*, this would raise the possibility of using non-lethal collection of tissue samples (e.g. leg clippings), thus avoiding the need to remove larval specimens from their natural habitat. Similar methods have been used on other insect species with minimal impact on survivorship or reproductive behaviour (Suzuki *et al*., 2012; Oi *et al*., 2013).

Most occurrence records for *Lucanus elaphus* are based on adult specimens, primarily males, that were attracted to electric lights. Staines (2001) used these and other collection records to produce a map showing the known distribution of *L. elaphus* in North America. Here, we go a step further by compiling precise occurrence records from diverse sources and then use them to build ecological niche models (ENMs), represented as geo-spatial projections of habitat suitability for *L. elaphus* across the eastern United States. Such models have been used to better understand the habitat associations of other saproxylic insects (Bosso *et al*., 2013), including lucanids (Thomaes *et al*., 2008; Huang, 2014).

Basic information on the ecology, genetics and geographic distribution of *L. elaphus* is presented here, with the former two components based on field work in two locations (Mississippi and Georgia). Taken together, these data are intended to provide a springboard for follow-up studies, and for formulating plans for assessing the conservation status of *L. elaphus* throughout its range.

Methods

Larval substrate associations and development

Between February 2014 and January 2015, searches for *L. elaphus* larvae were undertaken in mature closed-canopy hardwood-dominated forests on the Noxubee Wildlife Refuge in Oktibbeha and Winston counties, Mississippi (see coordinates in Appendix S1). The region experiences an average annual temperature of 16.9°C and an average annual rainfall of 140.2 cm (usclimatedata.com, accessed 12-Jan-2017). The search involved breaking open logs (i.e. fallen trees or branches) with a hand axe and/or inspecting the soil–log interface underneath. Any larvae belonging to the family Lucanidae (distinguished from scarab larvae by their longitudinal or Y-shaped anal openings) were collected. Data on log characteristics [e.g. diameter at point of collection, length, tree species (if recognisable) and rot type] were recorded from each log that yielded lucanid larvae. The findings from this effort focus only on logs within which larvae were found as it was not practical to search all logs thoroughly enough to confirm absence. Disturbance to breeding substrates was minimised by stopping the search within a log as soon as a lucanid larva was encountered. Moreover, when multiple larvae were simultaneously exposed within a log, only a single larva was collected unless the additional larvae appeared unlikely to re-establish within the disturbed substrate. All specimens were immediately weighed upon returning to the laboratory.

As it is not possible to reliably distinguish *L. elaphus* from other North American species of *Lucanus* based on larval morphology (Ritcher, 1966), all larvae collected in this study were raised to adulthood for identification. Modifying methods described by McMonigle (2004), a single batch of rearing substrate was prepared by mixing slightly decomposed oak chips with crumbly material collected from more thoroughly decomposed oak logs at an approximate ratio of 1:1. This material was dried in an oven for about 8 h at 102°C to reduce the incidence of potential invertebrate pests. Water was then added until the wood was completely saturated but not beyond the saturation point (i.e. water could be squeezed from the substrate but did not drip freely). Small plastic food-storage containers were filled to capacity with ~500 mL of this material and a single larva was added to each. Eight ~0.5 mm holes were poked through each lid to allow gas exchange while minimising water loss. The rearing containers were held at room temperature (~21–23°C) until adult emergence. They were checked daily to record dates of pupation and adult eclosion. Because most larvae formed pupation chambers at the bottom of their rearing container, these events could usually be observed with little disturbance. Voucher specimens of *L. elaphus* have been divided among the University of Georgia collection of Arthropods, the entomology collection at Mississippi State University and the research collection at the USDA Forest Service laboratory in Athens, Georgia. Voucher specimens of *Zelia vertebrata,* Say, a parasitoid of *L. elaphus* (see Results), are held in the personal research collection of J. O. Stireman III (JOSC), Wright State University in Dayton, Ohio.

A chi-square goodness-of-fit test was used to determine if the adult sex ratio deviated significantly from a 1:1 ratio. ANOVA was used on square root transformed data to compare the weights of male and female larvae at the time

of collection. For larvae collected in February to March of 2014 (which represented most of our specimens), comparisons in development time were made to determine how adult emergence patterns may differ between sexes. Because several of these individuals took over a year to complete development, data on time to pupation were not normally distributed. We therefore used the non-parametric Mann–Whitney *U*-test to compare the time required (in days) for the collected larvae to pupate in the laboratory. The same comparison was made using ANOVA after limiting the dataset to larvae that pupated within a year of being collected to get a better sense of differences in development time within the same adult cohort. Larval weight at the time of collection was initially included in this model as a potential covariate, but was subsequently omitted when found to be non-significant. The same analyses were used to compare the duration that males and females spent in the pupal stage.

Adult flight behaviour and timing of activity

A total of 60 flight intercept traps (consisting of two medially intersecting 20 × 30 cm clear plastic panels) were placed at two hardwood-dominated forested sites in Clarke County, Georgia [Whitehall forest (33°53′15.24″N 83°21′42.58″W) and Tallassee tract (33°58′41.91″N 83°29′23.77″W)]. The region experiences an average annual temperature of 16.9 °C and an average annual rainfall of 117.8 cm (usclimatedata.com, accessed 12-Jan-2017). Trapping at both sites took place within a few hundred meters of the Middle Oconee River. At each site, traps were placed at five locations on the forest edge and five locations at least 50 m into the forest interior. Each location received three traps suspended 0.5 m, 5 m and 15 m above the forest floor. The traps were baited with slow-release (15 mg day^{-1}) ethanol lures (Synergy Semiochemicals) and were filled with propylene glycol to kill and preserve the catch. Traps were emptied every 2 weeks for a period of 6 months beginning on 15 March 2016.

Genetics

To provide baseline data for potential use in DNA-based identification of *Lucanus elaphus*, a fragment of the mtDNA *COI* gene from 12 adults raised from larvae collected on the Noxubee Wildlife Refuge, Mississippi, was sequenced. Genomic DNA was extracted from adult leg clippings using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. The *COI* gene was amplified via polymerase chain reaction (PCR) in 10-μL volumes (or multiples thereof) comprised of 2.0 μL 5× PCR buffer (Promega, Fitchburg, WI, USA), 0.8 μL $MgCl_2$ (25 mM; Promega), 1.6 μL dNTPs (1.25 mM; Promega), 0.5 μL Bovine Serum Albumin (10 mg mL^{-1}; New England BioLabs, Ipswich, MA, USA), 3.0 μL dH_2O, 0.5 μL of each 10 μM primer (see Table 1), 0.1 μL Go-*Taq* (5U μL^{-1}; Promega) and 1.0 μL of genomic DNA. Amplifications were performed using a Bio-Rad T100 Thermal Cycler with the following profile: 95°C for 2 min (1 cycle), 95°C for 30 s, 48°C for 30 s, and 72°C for 1 min (35 cycles) and final extension of 72°C for 2 min (1 cycle). All amplified products were purified using ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA), and sequenced on an Applied Biosystems 3730× Genetic Analyser at Yale University (Foster City, CA, USA). Chromatograms were edited and aligned in MEGA v6.06 (Tamura *et al*., 2013). To confirm that data from true mtDNA had been obtained, sequences were translated into amino acids and compared to accessions in NCBI's nucleotide and protein databases via the BLAST search function (Altschul *et al*., 1990). Nucleotide composition and basic polymorphism summary statistics were calculated in DNASP v5.10 (Librado & Rozas, 2009). Phylogenetic relationships among unique haplotypes were estimated using statistical parsimony (Templeton *et al*., 1992) with the 95% confidence criterion enforced, implemented in TCS v1.21 (Clement *et al*., 2000). DNA sequences used in these analyses ($n = 11$ unique haplotypes) are publicly available in NCBI's GenBank database, under accession numbers KY026208-KY026219.

Geographic distribution

To identify geographic areas in which *Lucanus elaphus* has a high probability of occurrence, we estimated an ENM, using MaxEnt (Phillips *et al*., 2006). This was based on remote sensing–derived environmental data from 87 precise occurrence records. In addition to our own collections, species occurrence data were sourced from published literature (Staines, 2001), online public biodiversity inventory databases (i.e. Integrated Digitized Bio-collections, www.idigbio.org; Encyclopedia of Life, www.eol.org; and Insects of Iowa, www.insectsofiowa.com) and via personal communication with professional and citizen scientists (see Appendix S1 for complete details). GPS coordinates were either obtained directly, or estimated from specific location descriptions using Google Earth (www.google.com/earth). All low-resolution and redundant records were excluded as well as duplicate records where the locations fell within the same BioClim grid cell. The set of 87 locations were joined with BioClim variables and elevation data (SRTM Digital Elevation Model) from the WorldClim database (www.worldclim.org; Hijmans *et al*., 2005). Linear dependencies among the climate parameters can be a potential problem in ENM (Walker *et al*., 2009). The sensitivity of the MaxEnt models to possible lack of parameter independence was tested by comparing results generated using all 20 parameters, and subsets of 12, 9 and 7 parameters, where R-mode cluster analysis (after normalisation) was used to iteratively remove one of any highly correlated parameter pairs. The final ENMs were computed using the 12-parameter case, i.e. 11 of the available BioClim climate parameters plus elevation.

Table 1. PCR primer pairs used to amplify and sequence *Lucanus elaphus* mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I (*COI*) gene.

Target gene	Amplified product*	PCR primer name and sequence	Reference
COI	NUMT	LCO-1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ HCO-2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer *et al.* (1994)
COI	NUMT	C1-J-1718: 5′-GGAGGATTTGGAAATTGATTAGTT CC-3′ HCO2198: (as above)	Simon *et al.* (1994); Folmer *et al.* (1994)
COI	True mtDNA	LeCOI-F2: 5′-TTGGAAGATGATCAGGGATAGTCGG-3′ L2-N-3014: 5′-TCCAATGCACTAATCTGCCATATTA-3′	This study; Simon *et al.* (1994)

Asterisk: amplified products from most primer pairs were identified as being derived from a nuclear mitochondrial pseudogene (NUMT); bold text indicates the primer pair that reliably amplified products with DNA sequence characteristics of "true" mtDNA.

The entire 87 location dataset was modelled in MaxEnt using high-resolution 30-s (approximately 1 km at CONUS latitudes) climate and elevation grids. Together, the BioClim and elevation data characterise the overall temperature- and precipitation-related abiotic constraints on organismal distributions, and have been successfully used to model the niche of a species based on presence-only occurrence data (Richards *et al.*, 2007). Because preliminary analyses indicated that the ENM solution estimated from the full 87-record occurrence dataset resulted in erratic prediction of suitable habitat, two natural clusters of locations within the larger dataset were identified, and ENMs were estimated for each of them separately. These clusters were well-defined based on Q-mode cluster analysis as measured by complete-linkage Euclidean distance (which gave the highest cophenetic coefficient among the methods tested) using BioClim and elevation data sampled at a coarser 2.5-min resolution (approximately 5 km). The two-group set (the components labelled North and South, respectively) was then modelled in MaxEnt using higher-resolution 30-s grids. The resulting two independent ENM maps (Fig. S3b, c) were then combined (Fig. 3) using the following formula: $S_N + (1 - S_N) \times S_S$, where S_N and S_S represent environmental suitability, which represents the probability of a point occurrence (ranging from 0 to 1), for the North and South clusters, respectively.

Results

Larval substrate associations and development

Lucanid larvae were collected from 42 separate logs over the course of the study and larvae from 39 of these logs were successfully reared to adulthood. Of 75 total larvae collected, seven died from unknown causes, three (4%) were parasitised by *Zelia vertebrata* (Say) (Diptera: Tachinidae: Dexiini; see Discussion S1) and 65 were successfully reared to adulthood (including a few males which died as pupae). Completing development within 2 years of collection, the adult specimens consisted entirely of *L. elaphus* with 32 females and 33 males, consistent with a 1:1 sex ratio ($\chi^2 = 0.015$, 1 df, $P = 0.90$). The remaining logs, from which the presence of *L. elaphus* could not be confirmed, were excluded from further consideration. At collection, the weights of male and female larvae (1.88 ± 0.26 g vs. 1.67 ± 0.14 g) did not differ significantly ($F_{1,62} = 0.0$, $P = 0.99$). Most larvae weighed between 0.25 and 2.5 g when collected, and the distribution of weights appears largely unimodal (Fig. S1). Seven larvae weighed over 3 g, however, including one (Fig. 1d) that exceeded 6.5 g (Fig. S1).

All logs from which larvae were collected were hardwoods although most were too decomposed to be identified to genus. Unambiguous host records made during the survey include oak (*Quercus* spp., $n = 8$), beech (*Fagus grandifolia* Ehrh., $n = 1$) and Chinese tallow (*Triadica sebifera* (L.), $n = 1$). Larvae were recovered from a wide range of log sizes, ranging from logs 9–36 cm in diameter and from 0.5 to 17 m in length (Fig. S2). Larvae were found either tunnelling inside logs (24 logs) or feeding beneath logs at the soil–wood interface (15 logs; Fig. 1c–f). The species was found in association with a wide range of rot types without any noticeable preference, including white rot (Fig. 1e), brown rot (Fig. 1f) and even within veins of relatively intact wood surrounded by rot (Fig. 1d). All of the logs were highly decomposed but not thoroughly soft, almost always with some remaining areas of relatively hard wood. Logs yielding *L. elaphus* appeared to be largely undisturbed with a long history of direct soil contact (cf. logs moved by floodwaters). The logs were also characterised by very high moisture contents. The substrates within which *L. elaphus* were found feeding were always damp and sometimes thoroughly saturated. Drier wood, as sampled at upland sites or in logs with limited ground contact, never yielded *L. elaphus*. Many of the logs inhabited by *L. elaphus* were on the margins of regularly flooded areas that would have sometimes experienced standing water.

Although most larvae pupated within the year of collection, 16 individuals (25%), all males, postponed pupation for one additional year. Based on the Mann–Whitney *U*-test, females pupated more quickly than males ($Z = -4.5$, $P < 0.0001$) and also spent less time in the pupal stage ($Z = 3.4$, $P < 0.001$). To get a better sense of differences in development time within the same adult

cohort, the same comparison was made using the glm procedure of SAS (i.e. ANOVA on normally distributed data) after limiting the dataset to larvae that pupated within a year of being collected. These analyses also found females to pupate more quickly ($F_{1,40} = 10.4$, $P < 0.01$) and to spend less time in the pupal stage ($F_{1,34} = 13.0$, $P = 0.01$). For adults belonging to the same first-year cohort, females pupated 8.6 days sooner (159.6 ± 1.6 vs 168.2 ± 2.2 days) than males and spent 2.4 fewer days in the pupal stage (28.6 ± 0.3 vs 31.0 ± 0.6 days).

Adult flight behaviour and timing of activity

The flight intercept traps yielded a total of six adult *L. elaphus*, all from Tallassee Tract and all males. All specimens were captured at the forest edge and only in three of the five traps placed at 15 m above the ground. Dates of capture were as follows: 1 specimen 24 May to 7 June; 1 specimen 21 June to 7 July and 4 specimens 7–19 July 2016.

Genetics

When targeting the *COI* gene for PCR amplification from *L. elaphus* genomic DNA, we found that commonly used primer pairs (e.g. Folmer *et al*., 1994) co-amplified nuclear mitochondrial pseudogenes. This was evident from apparent heterozygous sites in sequence chromatograms (i.e. two or more peaks at a single position), some of which generated premature stop codons when translated. The issue was overcome by designing a species-specific forward primer (LeCOI-F2, see Table 1) based on information obtained from the subset of initial sequences that lacked heterozygous sites, coupled with diluting the template genomic DNA down to the lowest usable concentration. The resulting sequences had the following characteristics of true mtDNA: they were AT-rich (63.9%), they showed a bias towards transversion mutations (ts/tv ratio ratio = 7.1), no premature stop codons were detected, the majority of substitutions were synonymous and BLAST searches returned close matches to *COI* from *Lucanus* and other Coleoptera.

Based on 12 *L. elaphus* adults from Noxubee Wildlife Refuge, Mississippi, the 1364-bp mtDNA sequence alignment consisted of 11 unique haplotypes (Fig. 2). The alignment contained 39 segregating sites of which 13 were parsimony-informative sites, and mean, minimum and maximum uncorrected *p*-distances among haplotypes were 0.006, 0.001 and 0.013, respectively. Estimated phylogenetic relationships showed that although haplotypes are all closely related, at least two mutational changes separated each from its closest relative. Furthermore, there was no obvious clustering of haplotypes into two or more discrete clades on the network (Fig. 2), consistent with expectations for local panmixia.

Geographic distribution

Of the 12 parameters used in the final MaxEnt models, no single factor dominated in importance (Appendix S2). In the two-cluster partition of occurrence records, the three most important parameters in the model for the northern group were 'mean temperature of coldest quarter' (31.5%), 'precipitation of driest quarter' (19.9%) and 'precipitation of the warmest quarter' (17.1%). Those for the southern group were 'precipitation of driest quarter' (38.2%), 'precipitation of the coldest quarter' (21.5%) and 'minimum temperature of coldest month' (21.3%). Based on our combined map (Fig. 3), there appear to be three regions of relative high habitat suitability (i.e. coded as yellow or orange on the map). These regions seem to be separated by mountain ranges, with areas of low suitability corresponding to the Ozark and Ouachita mountains in northwestern Arkansas and southcentral Missouri and the Appalachian mountains which extend from northern Alabama into the northeastern states (Fig. 3).

Discussion

Our larval collection records indicate that *L. elaphus* breeds within or beneath decomposing hardwood logs varying widely in diameter and rot characteristics. Moreover, *L. elaphus* appears to be largely limited to hardwood-dominated stands near streams, including areas experiencing saturated soil and intermittent flooding, and are associated with damp or even saturated woody substrates. Our ENMs further suggest that *L. elaphus* is primarily associated with lowland forests with areas of relative low habitat suitability corresponding to the Ozark, Ouachita and Appalachian mountains. As additional occurrence records become available the resolution of ENMs can be further refined, allowing a closer examination of this pattern. To facilitate this, we provide the coordinates used in our analysis in Appendix S1.

It is noteworthy that no other lucanid species besides *L. elaphus* was collected in this study, raising questions about the habitat associations of the other species native to eastern North America. Most notably, not a single specimen of *L. capreolus* (L.) was encountered in our sampling despite it being the most commonly encountered *Lucanus* species throughout much of the eastern United States. The entomology collection at Mississippi State University, for example, contains 191 Mississippi specimens of *L. capreolus* compared to only 79 *L. elaphus* and 11 *L. placidus* Say (T. Schiefer, pers. comm., August 2015). These findings suggest that *L. elaphus* and *L. capreolus* associate with different forest types or utilise different substrates. Some species of *Lucanus*, including *L. cervus* in Europe and *L. placidus* in North America, are known to focus their feeding on roots and other subterranean structures (Milne, 1933; Ratcliffe, 1991; Harvey *et al*., 2011a). This may also be the case for *L. capreolus*,

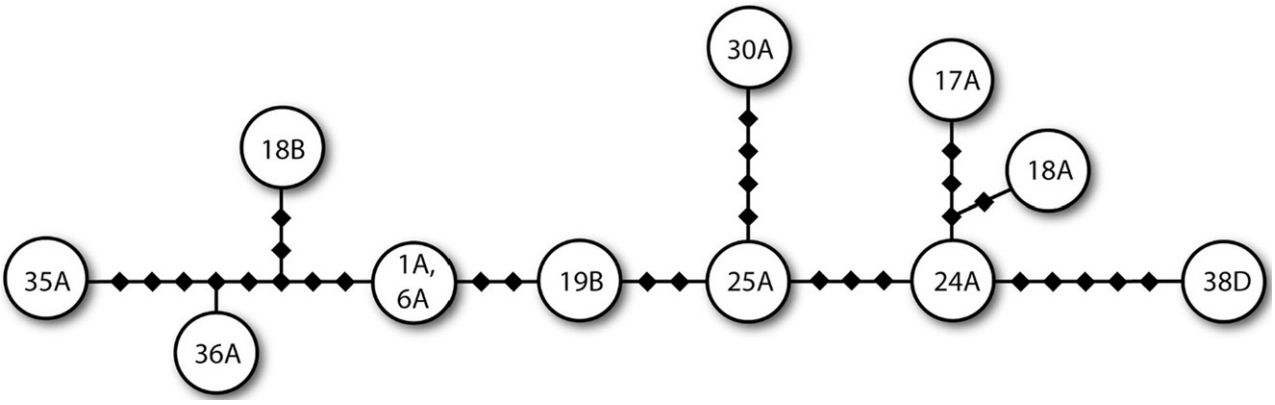

Fig. 2. Statistical parsimony network calculated in TCS (Clement *et al*., 2000) showing relationships among mitochondrial cytochrome *c* oxidase subunit I (*COI*) sequences from a subset of *Lucanus elaphus* specimens included in this study. Each unique 1364-bp haplotype is represented by a circle, and labelled with the identification code of the beetle(s) from which it was obtained. Black diamonds are hypothetical (i.e. unsampled or extinct) haplotypes, and each black line between any two haplotypes represents one mutational difference.

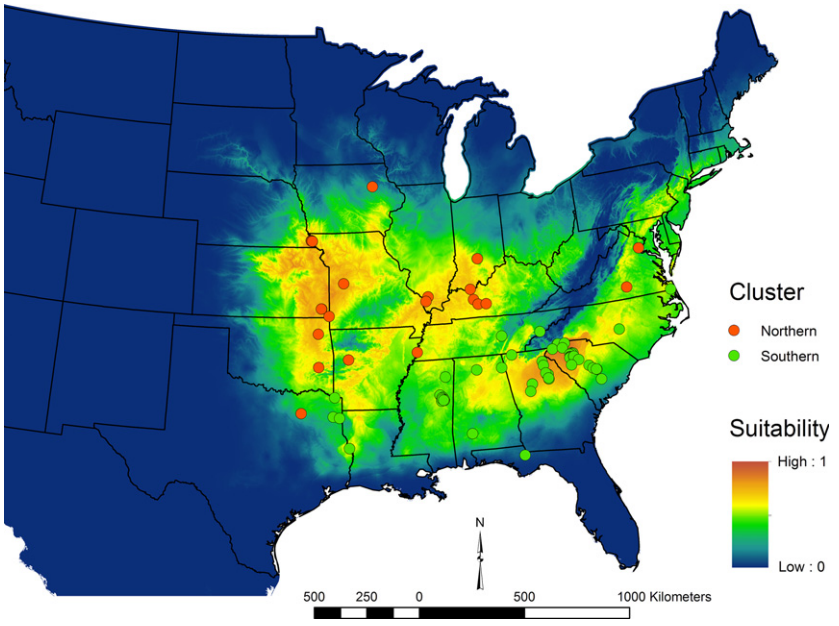

Fig. 3. Ecological Niche Models (ENMs) estimated for *Lucanus elaphus* using MaxEnt (Phillips *et al*., 2006). Habitat suitability is colour-coded from high (red) to low (dark blue). [Colour figure can be viewed at wileyonlinelibrary.com]

possibly explaining why no specimens were captured in this study.

The distribution of *L. elaphus* larval weights at the time of collection appeared largely unimodal (Fig. S1), suggesting that there is one generation per season and that most larvae complete development within 1 year. Support for this comes from a study by McMonigle (2004), who presented a table (pg. 16) showing the average time required for captive *L. elaphus* to complete each life stage at 21°C. The author reported that approximately 51 weeks were required by *L. elaphus* to develop from an egg to an adult. It should be noted that the distribution of larval weights in this study is skewed to the right, however, due to seven larvae that weighed >3 g. (Fig. S1). This is probably due to some male individuals postponing development for an additional year as we observed rearing specimens to adulthood, similar to the 31% of *Lucanus miwai* Kurosawa that delayed pupation for 1 year in Taiwan (Huang, 2014). Indeed, 75% (9 of 12) of larvae ≥2.5 g and 86% (6 of 7) of larvae ≥3 g at the time of collection were males. This finding is not surprising given that the larger sex in sexually dimorphic

insect species typically develops more slowly (Teder, 2014).

Although females are under-represented in collections (Blatchley, 1910), they are probably no less common than males in nature, as indicated by the 1:1 sex ratio detected in this study. The rarity of females in collections likely reflects differences in behaviour between sexes. Male *L. cervus* are known to fly more readily than females (Rink & Sinsch, 2007) and this may also be true for *L. elaphus*. Indeed, male *L. elaphus* are more commonly attracted to lights and all adult *L. elaphus* specimens captured in flight intercept traps in this study were males. Our flight intercept trap data suggest that male *L. elaphus* may be especially active high above the ground at the forest edge. This may be related to adult *L. elaphus* feeding on sap flows as many sap-feeding insects are known to be more abundant high above the ground in temperate deciduous forests (Ulyshen, 2011). This may also explain the apparent attractiveness of the slow-release ethanol lures attached to the traps as alcohols are produced by fermenting sap.

The occurrence of nuclear mitochondrial pseudogenes in *L. elaphus* is not unprecedented (e.g. Sunnucks & Hales, 1996; Bensasson *et al*., 2001). In fact, there have been indications of the same phenomenon in other *Lucanus* species. For example, in study of western Palaearctic stag beetles, Cox *et al*. (2013) reported that several of their mtDNA *COI* sequences had multiple heterozygous sites. We found that primer redesign and DNA template dilution was effective, but future mtDNA-based studies of *L. elaphus* should be cognizant of potential issues.

Generally speaking, the existence of 11 unique mtDNA *COI* haplotypes from 12 individuals sampled from the same location suggests that intra-specific polymorphism may be relatively high. Furthermore, given that the most divergent *L. elaphus* haplotypes in our set (1.3% divergent, uncorrected for multiple hits) were not partitioned into separate haplogroups (Fig. 2), these data suggest local panmixia and large effective population size (*Ne*) at Noxubee Wildlife Refuge, Mississippi. This large *Ne* could be the result of a very large census population size (*Nc*), such that even if only one tenth of individuals contribute genetic material to the next generation (Frankham, 1995), this fraction still represents many contributors. In addition, large *Ne* could be the result of incoming gene flow sourced from neighbouring regions.

There are only four described species of *Lucanus* found in the New World, and two of them (i.e. *L. capreolus* and *L. placidus*) have at least partly overlapping distributions with *L. elaphus*. Some *COI* sequence data are available for *L. placidus* (i.e. one 579-bp haplotype from specimens collected in Ontario, Canada; GenBank accessions: KM849664 and KM848696), and so it is possible to perform a preliminary assessment of Hebert *et al*.'s (2004) suggestion that *COI* barcoding is effective at discriminating among species when among-taxon sequence divergences are 10 times greater than the average within-taxon variability (i.e. the '10× rule', but see Hickerson *et al*., 2006). The mean uncorrected sequence divergence among the 11 *L. elaphus* haplotypes was 0.6%. Comparison of the two most divergent *L. elaphus* haplotypes with the haplotype available for *L. placidus* showed that uncorrected inter-specific divergences ranged from 16.1% to 16.4%. Inter-specific sequence comparisons were also possible for *L. mazama*—another North American *Lucanus* species for which some *COI* data are available (Sheffield *et al*., 2009; GenBank accession: FJ613419). Although *L. mazama* does not geographically overlap with *L. elaphus,* similar outcomes were obtained: *L. elaphus* differs from *L. mazama* by 15.2–15.7% sequence divergence (in this comparison, the full 1364-bp alignments could be used). Although this preliminary assessment is encouraging, the geographic scope of *L. elaphus* collections used for genetic analyses in this study was narrow. It is not uncommon for intra-specific *COI* polymorphism to exceed 1.5% sequence divergence in insects (Cognato, 2006). Indeed, Cox *et al*. (2013) found that while several species of western Palaearctic *Lucanus* could be distinguished on the basis of their *COI* sequence, others could not. Thus, geographically representative sampling and sequencing of *L. elaphus*, *L. capreolus* and *L. placidus* are needed.

Conservation implications

Although the conservation status of *L. elaphus* remains unknown, our findings provide valuable insights into the habitat requirements of the species. We conclude that *L. elaphus* favours lowland floodplain forests where it breeds in moderately to highly decomposed hardwood logs ranging widely in size and rot type. Efforts to protect mature lowland hardwood-dominated forests and provide an abundant and continuous supply of decomposing hardwood logs will benefit *L. elaphus* throughout its range.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: doi: 10.1111/icad.12229:

Discussion S1. On *Zelia vertebrata* parasitising *L. elaphus*.

Figure S1. Frequency distribution of larvae by weight at time of collection. Only larvae collected from February to March of 2014 are included here.

Figure S2. Dimensions of logs yielding larval *L. elaphus*. Diameter was measured at the point of collection.

Figure S3. Ecological Niche Models (ENMs) estimated for *Lucanus elaphus* using MaxEnt (Phillips *et al*., 2006).

Appendix S1. Occurrence records for *Lucanus elaphus* used for Ecological Niche Modelling.

Appendix S2. Relative contributions of the 12 variables (see definitions that follow) used in the Maxent models for the southern and northern clusters.

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